EVALUATING PROPHYLACTIC EFFICACY OF *TERMINALIA ARJUNA* (ROXB. EX DC.) WIGHT & ARN., *SANTALUM ALBUM* L., AND *NYMPHAEA X RUBRA* ROXB. EX ANDREWS FOR ACUTE MOUNTAIN SICKNESS

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Introduction

Acute mountain sickness (AMS) is the highest prevalent illness, occurring on rapid ascent to high altitude. The decreased partial pressure of oxygen at high altitude triggers a series of immediate physiological changes to cope with the hypoxic environment through the process of acclimatization. AMS typically precedes acclimatization and may even result in fatal conditions like high altitude cerebral and pulmonary edema, if neglected. Despite AMS being the most prevalent illness on acute exposure to high altitude, the pathophysiology of AMS still remains an enigma. The absence of clinical diagnostic markers and suitable animal models for AMS is probably the limiting factor for researchers worldwide in determining the molecular mechanisms resulting in AMS at high altitude. Though there have been several theories on physiological mechanisms leading to AMS, the matter continues to be debatable.

The exact pathophysiology of AMS is still not clear. There are many hypotheses about the pathophysiological mechanisms that lead to the symptoms of AMS. The fluid and electrolyte imbalance hypothesis of AMS predicts that the pathophysiology of AMS is partially attributed to increased sympathetic drive and dysregulation of renin-angiotensin-aldosterone system (RAAS) cascade (Swenson, 1997; Loeppky et al., 2003). It is well established that RAAS plays a prominent role in maintaining body fluid homeostasis (Xue et al., 2012). It not only controls the glomerular filtration rate but also maintains fluid and electrolyte balance in the body (Remuzzi et al., 2005). Further, RAAS-induced disruption of blood-brain barrier and increased intracranial pressure may lead to cerebral vascular leakage (CVL) contributing towards the pathogenesis of AMS (Ames et al., 1965; Purushothaman et al., 2008; Zhang et al., 2010). Among the most pivotal component of RAAS in Angiotensin-II, an antidiuretic hormone that, has been reported to decrease glomerular filtration rate and increase tubular reabsorption of sodium ions (Vos et al., 1992). Atrial natriuretic peptide (ANP), another hormone secreted from mammalian atria, regulates the homeostatic balance of body fluids, electrolytes and blood pressure (Laragh, 1985; Yasue et al., 1989). ANP stimulates vasodilatation, increases diuresis, and blocks the release and/or actions of several hormones, including angiotensin II, aldosterone and vasopressin (Nakayama et al., 1984; Baxter et al., 1988). The correlation of ANP with AMS has been controversial with some researchers reporting direct correlation, while others advocating an inverse correlation (Milledge et al., 1989; Tunny et al., 1989). Thus, understanding the effect of high altitude exposure on ANP mediated modulation of RAAS to prevent acute hypobaric hypoxia induced AMS is of utmost importance.

Increased brain volume and elevated intracranial pressure result in amplified blood-brain barrier (BBB) permeability that finally leads to augmented CVL, often referred to as 'tight fit hypotheses of symptoms associated with AMS' (Julian et al., 1985; Wilson and Milledge, 2008). BBB, formed by the brain endothelial cells, plays a dominant role in maintaining fluid homeostasis by regulating the flux of fluids and substances between the systemic circulation and brain microenvironment and protecting the brain from harmful xenobiotics that may cause damage to neuronal and non-neuronal cells present in brain microenvironment (Julian et al., 1985; Abbott, 2002). Acute exposure to hypobaric hypoxia has been well reported to cause disruption of BBB permeability and is associated with increased CVL (Song et al., 2016). Hypobaric hypoxia-mediated increased neuroinflammation has been reported to play a role in acute hypobaric hypoxia-induced altered BBB permeability (Julian et al., 1985). Under physiological conditions, homeostasis between pro- and anti-inflammatory cytokines plays a pivotal role in maintaining the immune responses in the body (Jeon and Kim, 2016). However, under pathophysiological conditions, dysregulation of homeostasis between pro- and anti-inflammatory cytokines leads to neuroinflammation and disruption of BBB (Wang et al., 2015).

S100 classes of proteins are the low molecular weight proteins, which have been characterized by the presence of two calcium binding sites with helix-loop-helix conformation (Marenholz et al., 2004). 21 different types of S100 class of proteins has been reported in the literature and considered as damage-associated molecular patterns (Memari et al., 2015). The major function of S100 class of proteins is sensing Ca2+ & once activated they may interact with other proteins resulting in regulation of their activity. S100B, one of the first identified S100 class of protein, has been reported to be secreted by astrocytes (Sorci et al., 2010). Secreted S100B protein may exert regulatory activities on intra- and extracellular signals and has been considered as a specific marker of brain injury (James et al., 2010; Sun et al., 2013). More specifically elevated serum S100B has been considered as a marker of neuroinflammation associated with acute and chronic injury (Michetti et al., 2012). High serum S100B protein levels have been found in subjects exposed to acute hypobaric hypoxia with symptoms of AMS (Winter et al., 2014). The receptor for the advanced glycation end products (RAGE), could work as signal traducer receptor and causes microglia activation resulting in neuroinflammation through NF-κB dependent mechanisms (Ramasamy et al., 2011). Interestingly, dissociated NF-κB has been found to control hundred of genes and has emerged to be a mediator of inflammatory processes (Aggarwal et al., 2011). So,

blocking of RAGE/NF-κB could be a therapeutic strategy for acute hypobaric hypoxia-induced neuroinflammation and subsequent CVL.

In addition to low partial pressure of oxygen, high altitude environment is also characterized by low absolute humidity. Though the existence hypothesis on AMS considerably focus on physiological changes associated with hypobaric hypoxia, the possible role of low humidity induced dehydration in triggering AMS has not been adequately explained in existence literature. Water is an essential nutrient and water deprivation of only 2% can result in impaired physical and performance (Kleiner, 1999). Dehydration is referred to as a state of deficiency in body water, which may be due to the excessive loss of body water and/or inadequate intake of water resulting in hypohydration in organs and tissues (Steiner et al., 2004). Previous reports suggest that dehydration results in fluid and electrolyte imbalance dependent increased plasma osmolality (Netti et al., 2014). Dehydration-induced increased plasma osmolality has been well reported to cause increase in red blood cell (RBC) fragility (Wu et al., 1998). Magnesium has been reported to play a dominant role in maintaining cellular functions and deficiency of magnesium has been associated with many of neurological and cardiac dysfunction (Swaminathan, 2003). In addition to this, there is a direct link of magnesium with endurance and performance. It has been reported that animals deficient in magnesium perform at a snail's pace in trade mill test (Keen et al., 1987). Magnesium also has a role in chronic fatigue syndrome. The patients with chronic fatigue syndrome have low red blood cells magnesium, further supporting the link of magnesium with performance and endurance (Cox et al., 1991). Exposure to dehydration has been previously reported to decrease serum magnesium concentration (Buchman et al., 1998). Previous studies report on shifting of magnesium ions from serum into RBC during marathon providing a plausible explanation for the decrease in serum Magnesium concentration (Lukaski et al 1983, Casoni et al 1990). Studies by De Franceschi et al., (1997) show that supplementation of magnesium reduces RBC dehydration in sickle cell disease. Thus, studying the effect of dehydration on RBC fragility and prevention of dehydration induced RBC fragility could indirectly mitigate the symptoms associated with AMS.

The prophylactics recommended are limited to acetazolamide, a potent carbonic anhydrase inhibitor, is the most commonly used and best-studied agent for the amelioration of AMS. The actual mechanisms by which acetazolamide reduces symptoms of AMS, however, remain unclear. Traditionally, acetazolamide's efficacy has been attributed to inhibition of CA in the kidneys, resulting in bicarbonaturia, metabolic acidosis and

respiratory system stimulation (Leaf and Goldfarb, 2007). However, acetazolamide is associated with many adverse effects viz., tinnitus, numbness, motor weakness, and fluid and electrolyte imbalance (Saito et al., 2011). Herbal nutraceuticals are known to provide health benefits including the prevention and/or treatment of a disease. Highly advantageous over allopathic medicines, the herbal formulations are free of side effects associated with allopathic medicines. Considering the plausible adverse effects of long term use of acetazolamide, there is a necessity for identifying alternative prophylactics preferably from herbal resources, to prevent occurrence of AMS on acute exposure to high altitude.

Terminalia arjuna (Roxb. ex DC.) Wight & Arn. *(T. arjuna)*, Terminalia arjuna (Roxb. ex DC.) Wight & Arn. (T. arjuna), better known as Arjuna, finds a mention in the book ‘Astang Hridayam' written by Vagbhatta around 1200 years ago (Dwivedi, 2007). Phytochemical analysis of *T. arjuna* bark extract reveals that, it contains triterpenes; viz., arjunolic acid, arjunic acid, arjunolitin and arjungenin, tannins; viz., arjunin, catechin, gallocatchin, epicatechin, epigallocatechin, glycosides; viz., arjunglucoside-I, arjunetoside , terminolitin, arjunetoside , 13 arjunaphthanoloside-1 and very high amount of flavonoids; viz., quercetin, kaempferol, luteolin, and 14 pellargonidin (Seth et al., 2013). *T. arjuna* bark extract has also been used in Ayurveda to treat renal failure (Das et al., 2010). In the traditional system of Ayurvedic medicine, *T. arjuna* has been used to treat angina pectoris and in recent ages, it is best known to have cardioprotective properties ranging from positive inotropic, hypolipidemic and coronary vasodilation (Dwivedi and Jauhari, 1997; Maulik and Katiyar, 2010). In addition, *T. arjuna* bark extract has been used in Ayurveda to treat renal failure (Das et al., 2010). However, information on the mechanism of action of *T. arjuna* is sparse in existing scientific literature for its potential to prevent hyperactivated RAAS-induced altered fluid and electrolyte balance.

Sandalwood, the dried heartwood of *Santalum album* L., which belongs to the family *Santalaceae*, has been known as green gold (Guo et al., 2014). It predominantly contains sesquiterpenes viz., α- and β-santalol with the percentage of 53 and 23 respectively (Misra and Dey, 2013). It has been reported in the literature to have sedative, anti-diarrohoeal, diuretic, antibacterial, and antiviral activities (Okugawa et al., 1995; Guo et al., 2014). In Ayurveda, a traditional Indian medical system, sandalwood has been reported as an anti-inflammatory drug and used traditionally to treat chronic inflammation of bladder and pyelitis (Kumar et al., 2015). Existence literature however does not report on the anti-

inflammatory and neuroprotective potential of sandalwood extract in acute hypobaric hypoxia induced neuroinflammation in rats.

Nymphaea x *rubra* Roxb. ex Andrews (N. rubra), with local name kumuda, is an aquatic perennial herb belonging to family *Nymphaeaceae*. Recently, it has been found that *N. rubra* is a hybrid produced by the cross between *N. lotus* and *N. pubescens* as a parental species (Dkhar et al., 2013). It grows on the shores of lakes, rivers and water bodies. The plant can grow to heights up to 6 to 7 feet in the water and has green colored leaves floating on water and pink colored flowers. The tubers are vertically covered with hairs. The plant is naturally found in Asia and in India mainly in Odisha and West Bengal (Behera et al., 2010). In the traditional medicine system, various parts of *N. rubra* have been used to treat various types of bleeding disorders (Sonowal and Baruna, 2011). In addition to this, pulverized rhizomes mixed with honey have been used as a therapeutic agent for bleeding nose, piles, dysentery and as cardiotonic (Yumnam et al., 2012). Pharmacologically, this whole plant is known to have protective effect on various disorders viz., anthelmintic, immunomodulation, insulin resistance, anti-hyperglycemic, anti-dyslipidemic, anti-inflammatory, anti-pyretic, hepatoprotective and free radical scavenging activity (Behera et al., 2010; Cheng et al., 2012; Rahuja et al., 2013; Gautam et al., 2014). The rhizomes of *N. rubra* have also been reported for their anti-oxidant efficacy. Total phenolic and flavonoids in the methanolic extract were found to be 0.36 and 0.67/100 g respectively (Daffodil and Mohan, 2014). Phytochemical analysis of flowers of *N. rubra* reveals the presence of polyphenolic compounds viz., rutin, quercetin, scopoletin and kaempferol (Gautam et al., 2014). Literature is sparse on extensive toxicological evaluation, phytochemical analysis, and therapeutic potential of *N. rubra* rhizome extract in dehydration-induced increased RBC fragility in rats.

The world's toughest, coldest, highest and bravest battle is being fought on Siachen Glacier for over the last 25 years. This is the most glaciated area outside the polar region comprising 22 glaciers. Troop deployment on the forward post is from 18, 000 ft. to 23, 000ft. AMS have been seen widely reported in the troops and these symptoms may be fatal if not treated timely. With the availability of scientific data on the use of *T. arjuna*, *N. rubra* and *S. album* for various ailments in traditional medicinal systems, their bio-safety is well established since ages. However, their implications in preventing causative factors for AMS viz., sodium retention, CVL, and hypovolemia have never been explored. No herbal formulation specifically preventing AMS in humans has either been reported in the traditional literature or modern medicine. Though some studies have been carried on plants like

Rhodiola rosea and *Panax ginseng* for high altitude-related ailments, their efficacy for preventing symptoms associated with AMS still remains debatable.

So, the present work was designed to investigate:

Objective 1. Development of an animal model for acute hypobaric hypoxia-induced alterations in fluid and electrolyte balance, cerebral vascular leakage, and hypovolemia in rats.

Phase 1. Development of an animal model to study the effect of acute exposure to hypobaric hypoxia on renal function and cerebral vascular leakage in rats.

Phase 2. To study the effect of dehydration on hematology, plasma volume, glomerular filtration rate, and body weight in rats.

Phase 3. To study the effect of combined hypoxia dehydration (CHD) stress on renal function, neuroinflammation, plasma volume, and hematology in rats.

Objective 2. Phyto-prophylactics for the prevention of Acute Mountain Sickness.

Phase 1. To study the effect of *Terminalia arjuna* bark extract on acute hypobaric hypoxia-induced decrease glomerular filtration rate in rats.

Phase 2. To study the effect of *Santalum album* heartwood extract on acute hypobaric hypoxia-induced cerebral vascular leakage and neuroinflammation in rats.

Phase 3. To study the effect of *Nymphaea rubra* rhizome extract on dehydration-induced increased RBC fragility in rats.

Objective 3. To study the prophylactic efficacy of polyherbal formulation comprising of *Terminalia arjuna, Santalum album* and *Nymphaea rubra* during CHD stress.

Phase 1. Preparation of polyherbal formulation.

Phase 2. Dose optimization and efficacy evaluation of polyherbal formulation in CHD model.

Phase 3. Toxicity evaluation of formulation.

Phase 4. Minimal optimal dose determination and efficacy evaluation in humans.

Objective 1.

Phase 1. Development of an animal model to study the effect of acute exposure to hypobaric hypoxia on renal function and cerebral vascular leakage in rats.

Materials and Method

Male Sprague-Dawley (SD) rats of body weight 220-260 g, were divided into seven groups, where group 1 animals consist of normal control animals maintained at the height of 1,198 ft., group 2, 3, 4, 5, 6 and 7 were simulated at 10,000, 15,000, 20,000, 27,000 and 30,000 ft. (24 animals per group with an exception of 6 animals per group for animals simulated at 30,000 ft.) altitude in the animal decompression chamber for 12 h. Glomerular filtration rate was estimated using ^{99m}Tc-DTPA, ANP, aldosterone, S100B, CVL, hematological profiling and electrolyte estimation. Serum ANP, aldosterone, and S100B concentration were measured using commercially available kits. Data was expressed as mean ± SD from three set of independent experiments and was analyzed using Graph Pad PRISM version 5.0 software (San Diego, California, USA). Data with multiple groups was analyzed by one-way analysis of variance (ANOVA) and posthoc analysis was performed using Newman-Keul's multiple comparison tests. Data with two experimental groups was analyzed by unpaired Student's t-test.

Results

We observed absolute mortality in animals exposed at the altitude of 30,000 ft. So, the rest of studies were limited to 27,000 ft. of hypobaric hypoxia exposure in the animal decompression chamber. Animals exposed at 27,000 ft. of hypobaric hypoxia showed a significant decrease in glomerular filtration rate as compared with normal control animals. However, apart from the animal exposed at 27,000 ft., none of the experimental animals showed significant difference in glomerular filtration rate as compared with normal control animals. In addition to this, serum ANP concentration was found to be increased in animals exposed to altitude of 15,000 ft. as compared with normal control animals up to the altitude of 15,000 ft. No significant difference in serum ANP concentration was observed in animals exposed to the altitude of 20,000 ft. as compared with normal control animals. In contrast to this, animals exposed to the altitude of 25,000 and 27,000 ft. Showed decrease in serum ANP concentration as compared with normal control animals. However, the serum aldosterone concentration was found to be decreased at the altitude of 15,000 ft. as compared with normal control animals. Animals exposed to higher altitude viz., 20,000, 25,000 and 27,000 ft.

showed altitude dependent increase in serum aldosterone concentration as compared to normal control animals.

The increase in serum S100B levels has been reported in the literature as a cardinal sign of neuroinflammation. Animals exposed to the altitude of 25,000 and 27,000 ft. showed increase in serum S100B concentration as compared with normal control animals. In the same line, CVL was found to be significantly increased in animals exposed at the altitude of 25,000 and 27,000 ft. as compared with normal control animals. However, no significant difference in any of hematological parameters was observed in animals exposed at 27,000 ft. when compared with normal control animals. The animals exposed at the altitude of 27,000 ft., however, showed a significant increase in serum sodium ion concentration and decrease in serum potassium ion concentration as compared with the normal control animals.

Phase 2. To study the effect of dehydration on hematology, plasma volume, glomerular filtration rate and body weight in rats.

Material and Methods

Male SD rats of body weight 220-260 g (n=6), were divided into two groups viz., group 1 normal control animals (food deprived) and group 2 dehydration control animals (3 days) with food and water deprivation for 3 days. These animals were then accessed for hematological parameters estimation, plasma volume estimation, and glomerular filtration rate estimation. Data was expressed as mean ± SD from three set of independent experiments and was analyzed using Graph Pad PRISM version 5.0 software (San Diego, California, USA). Data with two experimental groups was analyzed by unpaired Student's t-test.

Results

The increase in packed cell volume (PCV) and a decrease in plasma volume have been considered to be a cardinal sign of dehydration. In our studies, animals deprived of water for 3 days showed a significant decrease in PCV and plasma volume as compared to normal control animals. Dehydrated animals showed a significant decrease in glomerular filtration rate and body weight as compared with normal control animals. However, animals exposed to dehydration showed significant increase in red blood cell (RBC) concentration as compared to the normal control animals which was associated to decreased plasma volume.

Phase 3. To study the effect of combined hypoxia and dehydration (CHD) exposure on renal function, neuroinflammation, plasma volume and hematology in rats.

Material and Methods

Male SD rats of body weight 220-260 g (n=24), were divided into two groups viz., group 1 normal control animals (1,167 ft.) and group 2 were considered to be CHD control animals, were initially dehydrated for 60 hrs and then exposed to hypobaric hypoxia for 12 hrs. During 12 hrs of hypobaric hypoxia at the altitude of 27,000 ft, animals were also deprived of water, so considering it to be a CHD model. These animals were then accessed for hematological parameters viz., RBC, hemoglobin and PCV, plasma volume, glomerular filtration rate and serum S100B concentration estimation. Data was expressed as mean ± SD from three set of independent experiments and was analyzed using Graph Pad PRISM version 5.0 software (San Diego, California, USA). Data with two experimental groups was analyzed by unpaired Student's t-test.

Results

In this study when animals were exposed to CHD, they showed significantly increased S100B serum concentration and significantly decreased glomerular filtration rate as compared with normal control animals. Increased levels of PCV and decreased levels of plasma volume were observed in animals exposed CHD as compared with normal control animals. However, we have not seen any change in haematological parameters, when animals were exposed to acute hypobaric hypoxia for 12 hrs. When, animals were dehydrated for 72 hrs, we have seen increased PCV, decreased plasma volume and decreased glomerular filtration rate. But, when we exposed animals to CHD, we have seen the additive effect of dehydration on acute hypobaric hypoxia.

Objective 2.

Phase 1. To study the effect of *Terminalia arjuna* bark extract on acute hypobaric hypoxia-induced decreased glomerular filtration rate in rats.

Material and Methods

The red-colored bark of *T. arjuna* was collected from Subarnapur district of the state of Odisha, India in the month of October 2014 and was authenticated by, Dr. O. P. Chaurasia, an ethnobotanist at Defence Institute of High Altitude Research (DIHAR), Leh-Ladakh, India. Powered plant material was extracted with 70% ethanol using a Soxhlet apparatus. The solvent was removed under reduced pressure using rota-evaporator (BUCHI, Switzerland) and lyophilized (Labconco Freezone 4.5 Plus). The percentage yield of powdered extract was

approximately 27% weight/weight. *T. arjuna* bark extract was standardized using gallic acid, catechin, and arjunolic acid as a marker compound in reverse phase high-performance liquid chromatography (RP-HPLC) analysis (Infinity 1200 Series). Adult male SD rats weighing between 220-260 g were used in this study. Acute and sub-acute toxicity studies were done according to the organization for economic corporation and development (OECD) guidelines. For optimizing the dose to induce diuretic effect, lyophilized *T. arjuna* bark extract powder was dissolved in normal saline solution and orally administered to six animals per group at doses of 100 mg/kg, 150 mg/kg, 200 mg/kg, and 250 mg/kg *b. w.* (*p.o*). ANP concentration, glomerular filtration rate, and urine volume were estimated after 12 hrs of administration of a single dose of the extract and the results obtained were compared with vehicle (normal saline) treated animals. Animals exposed to hypobaric hypoxia simulating an altitude of 27,000 ft., in an artificially lighted specially designed animal decompression chamber with temperature and humidity maintained at 32±10C and 60% RH respectively. Male SD rats of weight ranging from 220-260 g, were randomly divided into six groups with 18 animals in each group. While animals in Group 1 received 2 ml normal saline and were kept in normoxic conditions, Group 2 normoxic animals were administered with hydro-alcoholic extract of *T. arjuna* at an oral dose of 150 mg/kg *b.w*. dissolved in 2 ml normal saline based on dose optimization. Group 3 comprised of *T. arjuna* administered animals (150 mg/kg *p.o*.) maintained under normoxic conditions that received anantin, an ANP receptor antagonist at a dosage of 10 µg intravenously. Group 4 comprised of hypoxic placebo while animals in Group 5 were administered with *T. arjuna* extract (150 mg/kg *p.o*.) 30 minutes prior to hypoxic exposure following which they were exposed to hypobaric hypoxia. Group 6 animals received 150 mg/kg of *T. arjuna* bark extract (*p.o*.) and 10 µg of anantin intravenously and exposed to hypobaric hypoxia after 30 minutes of drug administration hypobaric hypoxia. Histological studies were performed by hematoxylin and eosin staining for investigating changes, if any, caused during toxicological evaluation of *T. arjuna*. Urine volume was measured for individual rats in each group for 12 h during the period of exposure to hypobaric hypoxia using metabolic cages. For the hypoxic group, the metabolic cages were placed inside the decompression chamber during the exposure to hypobaric hypoxia. Glomerular Filtration Rate was determined from the residual half-life of intravenously injected ^{99m}Tc-DTPA. Serum electrolytes were estimated by flame photometry. Serum renin, angiotensin-II, aldosterone, and ANP were estimated using ELISA kits according to the manufacturer's instructions. *In-silico* interactions studies of arjunolic acid present in the

extract were performed by using commercially available software Vlife MDS. Data was expressed as mean ± SD from three set of independent experiments and was analyzed using Graph Pad PRISM version 5.0 software (San Diego, California, USA). All parameters were analyzed by ANOVA and posthoc analysis was performed using Newman-Keul's multiple comparison tests.

Results

RP-HPLC fingerprinting reveals the presence of gallic acid, catechin, arjunolic acid along with other compounds in the hydro-alcoholic extract of *T. arjuna* bark extract. Anatomical and histological examination revealed no adverse effect on vital organs viz., brain lungs, kidney, spleen, liver, and heart. The minimal optimal dose for the hydro-alcoholic extract of *T. arjuna* was determined to be 150 mg/kg b.w., which was used for further experimentation. We observed an increase in ANP concentration, glomerular filtration rate and urine volume in normoxic rats administered with *T. arjuna* extract at doses above 150 mg/kg b.w. Exposure to hypobaric hypoxia resulted in significant decrease in urine volume in vehicle treated group, administration of *T. arjuna* extract 30 min. prior to hypobaric hypoxia exposure maintained urine volume close to normoxic rats. In addition to maintenance of urine volume, *T. arjuna* bark extract also ameliorated hypobaric hypoxia-induced decrease in serum potassium and increase in serum sodium, which was observed in vehicle-treated hypoxic animals. The plasma half-life of ^{99m}Tc provides an indirect measure of glomerular filtration rate. Nonlinear regression analysis for the half-life of ^{99m}Tc showed that acute exposure to hypobaric hypoxia simulating an altitude of 27,000 ft. resulted in significant increase in plasma half-life of ^{99m}Tc as compared to normoxic vehicle treated animals. Administration of *T. arjuna* bark extract prior to hypoxic exposure did not alter total protein concentration in the serum but decreased plasma half-life of ^{99m}Tc. *T. arjuna* induced increase in glomerular filtration rate was attenuated by administration of ANP-receptor antagonist, anantin. Exposure to hypobaric hypoxia also resulted in increased serum conc. of renin, angiotensin-II and aldosterone concentration in vehicle treated hypoxic groups, which was ameliorated by administration of *T. arjuna* bark extract prior to hypoxic exposure. *T. arjuna* extract, co-administrated with anantin to hypoxic animals, failed to reduce renin, angiotensin-II and aldosterone concentration in the serum indicating ANP mediated regulation of RAAS system resulting in increased glomerular filtration in *T. arjuna* administered hypoxic animals. Molecular docking studies of arjunolic and evaluation of inter-molecular interactions in terms of docking scores were done using Vlife MDS 4.4 Biopredicta module. Arjunolic acid

showed best orientations (LP 2, 3, 5, 8 and 10) of docked molecule. Residues present in ANP receptor viz., Gln246A, Ser215A, Pro163A, Val59A, Asp160A, Arg157A, and Asp156A are responsible for hydrophobic interactions. Studies reveal that LP5 pose of the predominant molecule binds deeply with ANP receptor.

Phase 2. To study the effect of *S. album* heartwood extract on acute hypobaric hypoxia-induced cerebral vascular leakage and neuroinflammation in rats.

Material and methods

S. album heartwood benzene extract (SAE) was provided as an exgratia sample by Ambe-Phytoextracts Pvt. Ltd. Delhi. Adult male SD rats weighing between 220-260 g were used in this study. Acute and sub-acute toxicity studies were conducted according to the OECD guidelines. For optimization of the dose, the percentage change in IL-6 levels in the rat brain hippocampal lysate was considered as the standard parameter. SAE was dissolved in the normal saline solution and orally administered to six animals per group at the doses of 75, 150 and 300 mg/kg b.w. Male SD rats weighing 220-260 gm were exposed to hypobaric hypoxia simulating an altitude of 27,000 ft., in an artificially lighted specially designed animal decompression chamber with temperature and humidity maintained at 32±10C and 60% RH respectively. Male SD rats (n=6) were divided into six groups, where group 1 animals represents normal control animals treated with normal saline solution, group 2 animals were normal control animals treated with SAE at the dose of 150 mg/kg only, group 3 animals were considered to be hypoxic control animals treated with vehicle, group 4 animals were acute hypoxic control animals treated with cardamonin, an NF-κB inhibitor, at the dose of 5 mg/kg *p.o*., group 5 animals were hypoxic animals treated with RAGE inhibitor, FPS-ZM1 at the dose of 5 mg/kg *i.p.* and group 6 animals were considered to be hypoxic animals treated with SAE at the dose of 150 mg/kg. Percentage change in CVL during hypobaric hypoxia was estimated by using sodium fluorescein dye as an indicator of CVL. Histological studies for investigating the adverse changes, if any, caused during toxicological evaluation of SAE was performed by hematoxylin and eosin staining. Percentage change in the expression of caspase-3 in CA3 region of the hippocampus was performed by immunofluorescence techniques. Percentage change in the expression of RAGE and NF-κB protein was studied by immunoblotting and percentage change in the levels of S100B, TNF-α, IL-1β, and IL-6 was performed by ELISA kits according to the manufacturer's guidelines. *In-silico* interaction studies of the α-santalol present in the SAE was performed by using commercially available software Vlife MDS. Data was expressed as mean ± SD from three

set of independent experiments and was analyzed using GraphPad PRISM version 5.0 software (San Diego, California, USA). Data was analyzed using one-way analysis of variance followed by Neuman Keuls posthoc test and $p<0.5$ was considered to be statistically significant.

Results

Anatomical and histological examination revealed no adverse effect on vital organs viz., brain lungs, kidney, spleen, liver, and heart. The optimal dose for SAE was determined to be 150 mg/kg b.w., based on hippocampal IL-6 levels and CVL during hypoxia and this dose was used for further experimentation. In the present study, acute exposure to hypobaric hypoxia resulted in increased expression of the S100B protein in the hippocampus as compared to normal control animals. However, treatment of hypoxic animals with NF-κB blocker and RAGE receptor blocker did not alter the expression of S100B protein expression in rat brain hippocampus. In addition to this, administration of SAE to hypoxic animals did not alter the expression of S100B protein in rat brain hippocampus. In the present study, none of the experimental groups showed a significant change in the expression of RAGE receptor in rat brain hippocampus. NF-κB expression was found to significantly high in hypoxic animals as compared to normal control animals. However, treatment of animals with RAGE receptor blocker and SAE significantly reduces the expression of activated NF-κB as compared to hypoxic animals. No significant difference in expression of NF-κB was observed in animals treated with the inhibitor of NF-κB as compared to hypoxic animals. Acute exposure to hypobaric hypoxia was found to increase the levels of pro-inflammatory cytokine IL-6 in rat brain hippocampus as compared to normal control animals. However, treatment of hypoxic animals with inhibitors of RAGE and NF-κB significantly attenuates acute hypobaric hypoxia-induced increase in pro-inflammatory cytokines in rat brain hippocampus as compared to hypoxic control animals. Molecular docking studies of α-santalol and evaluation of inter-molecular interactions in terms of docking scores were done with respect to the reference ligand, Maltotriose, using Vlife MDS 4.4 Biopredicta module. α-santalol showed best orientations (LP3, 4, 7, 8 and 10) of docked molecule. Residues present in RAGE viz., Lys42N, Asp41N, Tyr155N, and Glu153N are responsible for hydrophobic interactions. Studies reveal that LP7 pose of the predominant molecule binds deeply and shows 40% of similarity with external ligand of RAGE receptor.

Acute exposure to hypobaric hypoxia was found to significantly increase levels of activated NF-κB in rat brain hippocampal lysate as compared to normal control animals.

Administration of SAE to hypoxic animals significantly attenuated acute hypobaric hypoxia-induced increase in NF-κB levels in rat brain hippocampal lysate as compared with hypoxic control animals. However, no significant difference in RAGE expression was observed in any of experimental groups. Administration of SAE to hypoxic animals significantly also attenuated acute hypoxia-induced increase in pro-inflammatory cytokines viz., IL-6, IL-1β, TNF-α levels, CVL, and serum S100B levels in the rat as compared with hypoxic control animals.

Phase 3. To study the effect of *Nymphaea rubra* on dehydration-induced increased RBC fragility in rats

Material and Methods

N. rubra rhizomes were collected in the month of November 2014 from Balangir district of the state of Odisha, India. The plant material was identified by a plant taxonomist at Sambalpur University, Odisha and the leaves and inflorescence were archived in the herbarium of DIHAR for future reference. Extraction of pulverized rhizomes of *N. rubra* was done using 100% methanol as a solvent in a Soxhlet apparatus. Phytochemical fingerprinting of lyophilized *N. rubra* rhizome extract was done for compounds previously reported to be present in the flowers viz., rutin, quercetin and caffeic acid by previously described protocol using RP-HPLC (Infinity 1200 Series). In-vitro cytotoxicity studies were conducted for doses of 0, 1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml of extract in N2a cell lines. The bio-availability study was done according to previously described protocols with minor modifications using quercetin as a reference standard. Six female animals were administered with a single dose of 200 mg/kg b.w., *p.o*. of *N. rubra* rhizome extract. Acute and sub-acute toxicity studies were performed according to the OECD guidelines and previously described protocols. For optimizing the dose of *N. rubra* rhizome extract, the powder was dissolved in normal saline solution and orally administered to six animals per group at doses of 100, 200 and 400 mg/kg, *p.o* and RBC fragility was measured. Male SD rats of body weight ranging from 220-260 g were randomly divided into four groups with six animals in each group. While animals in Group 1 received no treatment and were kept in normal conditions, Group 2 normal control animals were administered with lyophilized methanolic extract of *N. rubra* rhizome at an oral dose of 200 mg/kg b.w. in a pellet form with an oral gavage. Group 3 animals were dehydration control animals while Group 4 animals were administered orally with *N. rubra* rhizome extract in a pellet form during dehydration. The animals in Group 3 and 4 were subjected to water restriction for a period of

72 hrs to induce dehydration. The fresh blood samples collected were then analyzed for hematological parameters viz., RBC's, hemoglobin and PCV and plasma volume as estimated as described earlier. Based on previous reports, the increase in the PCV and decreased plasma volume was considered to be a marker of dehydration. RBC fragility was assessed according to the previously published methods with minor modifications.

Results

RP-HPLC fingerprinting reveals the presence of rutin, caffic acid, and quercetin along with other compounds in methanolic extract of *N. rubra* rhizome extract. MTT assay revealed that supplementation of N2a cells with *N. rubra* rhizome extract up to the dose of 31.25 µg/ml did not alter cell viability. The IC50 value calculated for *N. rubra* rhizome extract on N2a cell lines was determined as 500 µg/ml. Anatomical and histological examination revealed no adverse effect on vital organs viz., brain, lungs, kidney, spleen, liver, and heart. RP-HPLC analysis of serum samples prior to and 4 h after oral administration of *N. rubra* rhizome extract showed the presence of quercetin in post drug administration samples. Quercetin was however not detected in serum samples of rats prior to drug administration. The optimized dose for preventing dehydration induced increased RBC fragility was found to be 200 mg/kg of *N. rubra* rhizome extract. Exposure to dehydration results in increased RBC, PCV, and Hb in rats. Administration of *N. rubra* rhizome extract during dehydration did decrease the dehydration induced increased PCV and hemoglobin. Exposure to dehydration results in significant increase in osmotic fragility of RBC in rats when compared with normal control animals. Administration of *N. rubra* rhizome extract significantly attenuates the dehydration-induced increase in RBC fragility in rats.

Objective 3.

Phase 1. Preparation of polyherbal formulation.

Material and methods

T. arjuna, *S. album* and *N. rubra* were mixed in eleven different proportions, in order to optimize the exact composition of polyherbal formulation. Adult male SD rats weighing between 220-260 g (n=12) were used in this study. For optimization of the composition, experimental animals were divided in to four groups, where group 1 animals were considered to be normal control animals treated with saline solution, group 2 animals were normal control animals treated with different composition of polyherbal formulation, group 3 animals were CHD control animals treated with normal saline solution and group 4 animals were CHD control animals treated with different composition of polyherbal formulation.

Estimation of glomerular filtration rate was considered to be a standard parameter for optimization of *T. arjuna* composition, brain hippocampal IL-6 expression as a standard parameter for *S. album* and RBC fragility as a standard parameter for the optimization of the composition of *N. rubra* in polyherbal formulation.

Results

The optimal composition of *T. arjuna*, *N. rubra* and *S. album* in polyherbal formulation was found to be 1:1:0.25 respectively.

Phase 2. Dose optimization and efficacy evaluation of polyherbal formulation in CHD model.

Material and methods

The dose of the composition was estimated using CVL as a standard parameter in rats. Animals were divided in to seven groups where group 1 animals consist of normal control animals, group 2 animals were considered to be CHD control animals and group 3, 4, 5, 6 and 7 were considered to be CHD treated animals, treated with different doses of formulations viz., 150, 300, 600, 900 and 1200 mg/kg of body weight in the proportion of 1:1:0.25 for *T. arjuna*, *N. rubra* and *S. album* respectively. After the treatment, animals were sacrificed and CVL was estimated by using sodium fluorescein dye as an indicator of CVL.

Results

During CHD exposure to normal control animals, the CVL was found to significantly higher as compared with normal control animals. No significant difference in CVL was found in animals treated with 150 mg/kg of polyherbal formulation as compared with CHD control animals. There is a significant difference in CVL was observed in animals treated with 300 mg/kg of polyherbal formulation as compared with CHD control animals. There was also significant difference in CVL was observed in 600 mg/kg and 300 mg/kg of polyherbal formulation treated animals. But there was no significant difference in CVL was observed in animals treated with 600 mg/kg as compared with 900 mg/kg of polyherbal formulation treated animals. So, the standard optimal dose of the polyherbal formulation was found to be 600 mg/kg.

Phase 3. Toxicological evaluation of polyherbal formulation.

Material and methods

Acute and sub-acute toxicity studies were performed according to the OECD guidelines described earlier.

Results

Anatomical and histological examination revealed no adverse effect on vital organs viz., brain lungs, kidney, spleen, liver, and heart. The NOAEL of polyherbal formulation was found to be less than 2000 mg/kg.

Phase 4. Minimal optimal dose determination and efficacy evaluation in humans.

Material and methods

Human equivalent dose of *T. arjuna, N. rubra and S. album* was calculated by the formula for the guidelines provided "Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers". Condiments viz., Dhania (*Coriandrum sativum*) fruit, Clove (*Syzygium aromaticum*) flower buds, Black pepper *Piper nigrum* seed powder, Badi elaichi (*Amomum subulatum*) seed powder, fruit of Nutmeg (*Myristica fragrans)* and edible Karpur (*camphor*) powder mixed in different proportions in order to obtain organoleptic score greater than 8. Human studies were conducted on volunteers staying at Leh (11,300ft) who were inducted to an altitude of 17,680 ft. (very high altitude) at DRDO Research Station, Chang La. Volunteers (n=5/group) were categorized into 03 groups and were supplemented with viz., placebo, polyherbal formulation at the dose of 600 mg each four times before induction to very high altitude and single acetazolamide (250 mg) treated subjects. Number of micturations, RBC fragility and Lake Louise Scores were measured at intervals of 12 hrs, 24 hrs and 36 hrs of induction.

Results

The human equivalent dose of *T. arjuna, N. rubra and S. album* has been found to be 37.8, 37.8 and 9.3 mg/kg respectively. The optimized composition of condiments Condiments viz., Dhania (*Coriandrum sativum*) fruit, Clove (*Syzygium aromaticum*) flower buds, Black pepper *Piper nigrum* seed powder, Badi elaichi (*Amomum subulatum*) seed powder, fruit of Nutmeg (*Myristica fragrans)* and edible Karpur (*camphor*) powder was found to be 1:0.5:0.5:0.5:0.3:0.5 respectively.

During the initial 12 hrs of very high altitude exposure, the placebo subjects showed lower number of micturations, increased RBC fragility, higher Lake Louis Score and percentage prevalence of AMS was found to be 80%. The symptoms associated with AMS were found to decrease during 24 and 36 hrs of stay at very high altitude. However, the subjects treated with polyherbal formulation and acetazolamide showed significant improvement in micturtions as compared with placebo treated subjects during 12, 24 and 36 hrs of stay at very high altitude. But the number of micrutations was found to be high in

formulations treated subjects as compared with the acetazolamide treated subjects. In the same line, in subjected treated with polyherbal formulation and acetazolamide showed decreased RBC fragility, lower Lake Louis Score as compared with placebo treated subjects. The percentage prevalence of AMS was found to be 0% in both, polyherbal formulation and acetazolamide treated subjects. But the efficacy of polyherbal formulation in terms of increasing micturations, decreasing RBC fragility and Lake Louise Score was found to be high as compared with acetazolamide treated subjects.

Salient findings of the study

1. Acute exposure to hypobaric hypoxia has been found to decrease glomerular filtration rate at the altitude of 25,000 ft. and 27,000 ft. and a decrease in glomerular filtration rate has been associated with decreased arterial natriuretic peptide levels and increased serum aldosterone levels. The decrease in glomerular filtration rate has been well associated with alterations in fluid and electrolyte balance, supporting fluid and electrolyte imbalance theory of AMS. In addition to this, serum S100B protein levels were found to increase with the increase in altitude along with increase in percentage prevalence of CVL. Increased serum S100B levels could be an indicator of neuroinflammation and further leads to BBB permeability and CVL, supporting the role of vasogenic edema theory of acute mountain sickness. We have also studied the effect of dehydration on various serological parameters in order to find out the correlation between dehydration and the symptoms associated with acute mountain sickness. Increased pack cell volume has been reported to be a cardinal sign of dehydration. During CHD, increased pack cell volume along with decreased glomerular filtration rate and increased serum S100B protein levels were observed. This indicates dehydration to be an additive factor for the symptoms associated with acute mountain sickness.

2. To summarize our findings, this is the first report demonstrating the adaptogenic and prophylactic potential of *T. arjuna* bark extract for high altitude maladies. *T. arjuna* bark extract ameliorates hypobaric hypoxia-induced decrease in glomerular filtration rate and sodium ion accumulation in the serum through ANP induced modulation of RAAS. So, *T. arjuna* could be beneficial in acute hypobaric hypoxia induced decreased glomerular filtration rate and altered fluid and electrolyte balance. This is also the first report demonstrating the anti-inflammatory role of *S. album* extract. S100B protein has been found to play a dominant role in acute hypobaric hypoxia induced neuroinflammation and altered BBB permeability and CVL in rats. Secreted S100B protein could lead to neuroinflammation through RAGE/NF-κB pathways. Preventive administration of SAE to hypoxic animals,

significantly reduces the activation of NF-κB-p65 signal cascade protein in the hippocampal lysate, supporting the role of SAE in reducing neuroinflammation. *In-silico* studies reveal, α-santalol present in *S. album* binds to RAGE receptor and could block RAGE receptor, thus preventing neuroinflammation during acute hypobaric hypoxia. This is the first study, demonstrating the potential of *N. rubra* in dehydration-induced increased RBC fragility in rats. Administration of the extract during dehydration decreased RBC fragility and improved serum magnesium concentration thereby attenuating the adverse effects of dehydration. Future studies on molecular mechanisms pertaining to *N. rubra* induced maintenance of structural integrity of RBC during dehydration could provide further support for its use as a prophylactic and therapeutic in dehydration and heat stroke-related conditions.

3. The polyherbal formulations developed by using *T. arjuna*, *N. rubra* and *S. album* was found to be highly effective in terms of symptoms associated with AMS. The efficacy of the polyherbal formulation is even higher than the acetazolamide.

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Participations:

- Participated in, 2nd IBRO/APRC Chandigarh Neuroscience School, Panjab University, Chandigarh, December 14-22nd, 2016.
- Participated in, ZOOCON-2016, themed, Integrating Biodiversity for Rephrasing Therapeutics and Sustainability, organized by Department of Zoology, Panjab University, Chandigarh, November 17-18th, 2016.
- Participated in, "Advanced Microscopy and Imaging Technique" jointly organized by, DSS Imagetech. Pvt. Ltd., Olympus Medical Systems India Pvt. Ltd. and Photometrics (USA) along with INST, Chandigarh from 16-17th January, 2015.
- Participated in, XXXII Annual Conference of Indian Academy of Neurosciences held at, National Institute of Mental Health and Neurosciences, Bengaluru, November 1-3, 2014.
- Participated in Neurologic Clinic at LIP, Leh, Jammu and Kashmir, India in collaboration with Cleveland University, USA from 15 of June to 19 of June, 2014.
- Participated in, 27th Annual Meeting of Society for Neurochemistry, India (SNCI) and International Conference-2013 on "Recent Advances in Molecular Mechanisms of Neurological Disorders" held at AIIMS, Delhi.
- Participated in, "Workshop on Drug Information Database and Its Applications in Pharmaceutical Research" organized by, ISF College of Pharmacy, Moga, Punjab, India on 4th May, 2013.
- Participated in, 64th Indian Pharmaceutical Congress, 2012 themed: "Pharmacy Education: Innovation, Strategies and Globalization" held at Chennai from, 7th December to 9th December, 2012.
- Participated in, "National Technology Day Convention-2012" held at ISF college of Pharmacy, Moga, Punjab, India on 7th of August, 2012.
- Participated in, "INDO-TAIWAN International Workshop 2011, themed: "Drug Development for Cancer and Infectious Disease from Basic to Industry" held at ISF College of Pharmacy, Moga, Punjab, India.
- Participated in, "APTI-16th Annual National Convection 2011" held at, ISF College of Pharmacy, Moga, Punjab, India from, 7- 9th of October, 2011.

Presentations:

- Presented a poster at, 2nd IBRO/APRC Chandigarh Neuroscience School, Panjab University, Chandigarh.
- Presented a poster presentation at, ZOOCON-2016, held at Department of Zoology, Panjab University, Chandigarh.
- Presented a poster at, "27th Annual Meeting of Society for Neurochemistry India (SNCI) and International Conference-2013, on "Recent Advances in Molecular Mechanisms of Neurological Disorders" held at AIIMS, Delhi.